

Effect of growth hormone on aging connective tissue in muscle and tendon: gene expression, morphology, and function following immobilization and rehabilitation

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Boesen AP, Dideriksen K, Couppé C, Magnusson SP, Schjerling P, Boesen M, Aagaard P, Kjaer M, Langberg H. Effect of growth hormone on aging connective tissue in muscle and tendon: gene expression, morphology, and function following immobilization and rehabilitation. *J Appl Physiol* 116: 192–203, 2014. First published November 14, 2013; doi:10.1152/jappphysiol.01077.2013.—It is unknown whether loss in musculotendinous tissue during inactivity can be counteracted by growth hormone (GH), and whether GH accelerate rehabilitation in aging individuals. Elderly men (65–75 yr; $n = 12$) had one leg immobilized 2 wk followed by 6 wk of retraining and were randomly assigned to daily injections of recombinant GH (rhGH; $n = 6$) or placebo (Plc; $n = 6$). Cross-sectional area (CSA), muscle strength (MVC), and biomechanical properties of m. quadriceps and patellar tendon were determined. Muscle and tendon biopsies were analyzed for gene expressions (mRNA) of collagen (COL1A1/3A1) and insulin-like growth factors (IGF-1Ea/Ec). Fibril morphology was analyzed by transmission electron microscope (TEM). In tendon, CSA and biomechanical properties did not change following immobilization, but an increase in CSA was found after 6 wk of rehabilitation in both groups. The changes were more pronounced when GH was injected. Furthermore, tendon stiffness increased in the GH group. Muscle CSA declined after immobilization in the Plc but not in the GH group. Muscle CSA increased during retraining, with a significantly larger increase in the GH group compared with the Plc group. Both a time and a group effect were seen for IGF-1Ea/Ec and COL1A1/3A1 mRNA expression in muscle, with a difference between GH and Plc. IGF-1Ea/Ec and COL-1A1/3A1 mRNA expression increased in muscle following immobilization and retraining in subjects receiving GH, whereas an increase in IGF-1Ec mRNA expression was seen in the Plc group only after retraining. In conclusion, in elderly humans, GH seems to have a matrix stabilizing effect during inactivity and rehabilitation by stimulating collagen expression in the musculotendinous tissue and increasing tendon CSA and stiffness.

growth hormone; connective tissue; tendon; skeletal muscle; inactivity

HUMAN AGING IS ASSOCIATED with a reduction in skeletal muscle mass (28, 34, 56), and human skeletal muscle force output drops ~1% per year after the age of 50 (72). It is also known

that physical activity is reduced with aging and that inactivity causes a loss in skeletal strength (6, 18, 56, 73). To what extent the loss in skeletal muscle with aging is a direct effect of the age-related decrease in activity, whether it is due to aging per se, or whether it is a mix of the two is at present unknown. Elderly individuals are in general more prone to periods of bed rest due to a higher degree of comorbidity and hospitalization (22, 48, 56), which may further accelerate the general loss of skeletal muscle mass and muscle strength (6, 18, 25, 52, 73). An accelerated loss of muscle mass and strength could thus be detrimental for the functional capacity and increase, e.g., the risk for fall injuries (27, 52). This underlines that maintenance of muscle mass and function in older individuals, especially during periods of inactivity, is of vital importance. Despite this, the effect of inactivity on skeletal muscle has mainly been investigated in young subjects (6, 10, 18, 26) and less frequently in elderly subjects (33, 41, 73). Recent short-term inactivity studies in the elderly have shown a reduced ability to regain lost muscle mass during recovery compared with young individuals (33, 73).

The tendon tissue may also be influenced by transient periods of immobilization or reduced loading. Animal studies have shown a decline of the mechanical properties of the tendon (2, 50, 65), whereas others suggest the opposite (3, 24). Recent studies in humans have shown that short-term inactivity has no major morphological effect on tendon size itself (17, 18, 69) but rather seems to decrease tendon biomechanical properties dramatically (17, 18, 69) and reduce collagen synthesis (14, 19).

The growth hormone (GH)/IGF-1 axis is known to play a central role in the regulation of human collagen turnover in musculotendinous tissue (21), with IGF-1 stimulating collagen formation in both tendon and skeletal muscle (1, 1, 59). IGF-I may also inhibit muscle protein degradation, which is an effect of potential importance during immobilization periods when there is a net muscle protein loss (80). Aging is associated with a gradual loss of the GH/IGF-I axis activity, and plasma levels of IGF-I decline with age (46, 64, 81). In fact, >30% of elderly men have IGF-1 levels below the reference range of young adults (46, 64). This decline in plasma IGF-1 levels is associated with a change in body composition, with increased body fat and a loss in muscle mass (35, 62). Administration of GH to elderly has been shown to increase plasma IGF-1 levels (49)

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and to cause gains in lean body mass (fat-free mass) (63). However, whether GH supplementation in elderly humans can counteract the loss in musculotendinous tissue quality and mass during inactivity or accelerate the effect of subsequent rehabilitation remains unknown. Studies on GH supplementation have generally failed to demonstrate any myofibrillar anabolic effect in the elderly (44, 74, 75). Interestingly, however, short-term treatment with GH or IGF-I in elderly women has been shown to induce elevated muscle protein synthesis and net whole body protein synthesis, respectively (11). In young humans, upregulation of skeletal muscle IGF-I expression is associated with increased collagen protein synthesis (20). These findings indicate a coupling between local IGF-I expression and collagen formation in human skeletal muscle.

In the present study, we hypothesize that systemic GH supplementation in elderly humans, via an increase in the local IGF-I level, will stimulate collagen expression of connective tissue of both the skeletal muscle and tendon tissue, and thereby affect the maintenance of connective tissue (collagen) and biomechanical properties of tendon during short-term inactivity and accelerate the aforementioned parameters during rehabilitation. Thus the aim of the present study was to investigate whether GH supplementation exerts a positive effect on the musculotendinous tissue in elderly humans during periods of short-term immobilization and rehabilitation.

METHODS

Study Design

Subjects. Twelve healthy, physically untrained elderly men (65–80 yr) with a body mass index between 19 and 30 were recruited for this study. All subjects underwent medical evaluation before participation, including a survey of their medical history and a physical examination, and all subjects included were healthy nonsmokers, and none took any kind of prescribed medication that affected skeletal muscle or tendon function. Furthermore, all subjects had no known serious injuries of their lower extremities. The study design was a double-blinded, randomized, placebo-controlled trial. The study (H-4-2010-010) was approved by the local Ethics Committee of Region Copenhagen in accordance with the Helsinki declaration. All subjects were informed of the risks associated with the study and gave their written, informed consent.

Intervention protocol. All participants were subjected to 2 wk of unilateral (randomly selected limb) lower limb cast immobilization from the hip to the ankle followed by 6 wk of supervised unilateral strength training (3 times/wk). All measurements described below were conducted at baseline before the immobilization procedure (Pre), after 2 wk of immobilization (Post immob), and again during reha-

bilitation (after 2 and 6 wk of heavy resistance training). Measurements in the training period were always performed with a “wash-out period” of 48 h after the last training session (Fig. 1).

rhGH administration. Subjects were randomly assigned in a double-blinded fashion to receive either rhGH (Norditropin, Novo Nordisk, Denmark) (rhGH; $n = 6$) or placebo (Plc; $n = 6$). rhGH/Plc was administered by daily subcutaneous injection in the proximal lateral part of the thigh (immobilized leg) for the 8-wk intervention period. rhGH dosage was $33.3 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ the first week, and if there were no signs of side effects the dosage was increased to $50 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ the remaining 7 wk. After detailed instructions, the subjects were able to perform the injections themselves at home. The participants visited the institute once every week throughout the intervention to ensure no side effects had occurred. If side effects were present (pitting leg edema, carpal tunnel syndrome, trigger fingers, weight gain or transient atrial fibrillation from fluid retention), the rhGH dosage was adjusted to a tolerable concentration. If possible thereafter, the dose was gradually increased again up to the intended dosage: $50 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$. Subjects performed the injections before bedtime, and blood samples were drawn early the next day during follow-ups.

Of the six subjects receiving rhGH, two subjects experienced mild side effects (both demonstrated mild carpal tunnel syndrome, and one of these trigger fingers), and the rhGH dose was reduced from $50 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ to $33 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ in these subjects until all side effects disappeared (~7 days). Subsequently, the dose was increased again to $50 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ with no discomfort.

Immobilization protocol. Immobilization was done for 2 wk using a lightweight fiber cast applied from just above the malleoli to just below the groin, which in other studies has proven to induce muscle atrophy in short-term immobilization studies involving elderly individuals (73). The cast was positioned in 50° of knee joint flexion to minimize walking ability using the casted limb, and the subjects were carefully instructed to perform all activities on crutches, to abstain from ground contact, and to avoid any contractile quadriceps activity in the immobilized leg. During the 2 wk of immobilization, the subjects were contacted every second day by mail or phone and were carefully instructed to do passive movements with the ankle joint (venous pump exercises) several times a day to prevent formation of deep venous thrombosis (DVT). Furthermore, to reduce the potential risk of DVT, all subjects took acetylsalicylic acid (ASA) tablets (75 mg/day) during the 2 wk of immobilization.

Rehabilitation procedure. After removal of the cast, the subjects received rehabilitation training for 6 wk, in three sessions per week (18 training sessions in total). The retraining protocol was a supervised, unilateral strength training program focusing on the immobilized leg only, which has previously proven to elicit increases in muscle size and maximal muscle strength in elderly individuals (73). After a 5-min warm-up on a stationary bike, the subjects performed supervised knee extension and leg press exercise. Training intensity and volume were 3–4 sets \times 12 repetitions [15 repetitions maximum

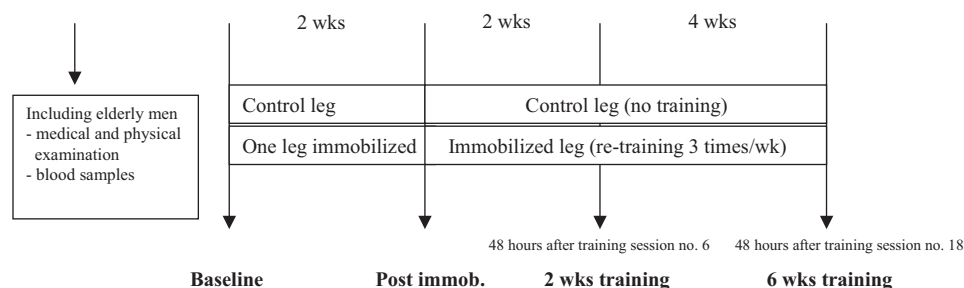


Fig. 1. Study design following 2 wk of immobilization and 6 wk of retraining in elderly men ($n = 12$) with either recombinant human growth hormone (rhGH; $n = 6$) or placebo ($n = 6$). Measurements performed at baseline after 2 wk of immobilization and again after 2 and 6 wk of retraining: blood samples, MRI scan [muscle and tendon cross-sectional area (CSA)], maximal isometric muscle strength, tendon biomechanical properties, biopsies (muscle and tendon; tendon biopsy was not performed after 2 wk of retraining).

(RM)] in week 1, 4 sets \times 10 repetitions (12 RM) in weeks 2–4, and 4 sets \times 8 repetitions (10 RM) in weeks 5–8. Training loads were adjusted on a weekly basis using 5-RM testing.

Measurements

Body composition. Dual-energy X-ray absorptiometry (Lunar DPX, version 3.6Z software) was used to determine whole body composition and percent body fat at baseline and again after 8 wk of rhGH or Plc administration. The same investigator (K. Dideriksen) performed and analyzed all scans using the extended research analysis software provided by Lunar. Total and regional scans were divided into three compartments: lean body mass (LBM), fat mass (FM), and bone mineral content (BMC).

Skeletal muscle cross-sectional area. To assess anatomical skeletal muscle cross-sectional area (CSA), all participants were scanned in a 1.5-T Philips Intera (Eindhoven, Holland) or a General Electric (GE) Signa Horizon MRI scanner using the following protocol. With the subject in the supine position and both limbs extended and relaxed using the body array coil, a scout localizer centered mid-femur was performed to control that the femurs and knee joints were included in the scanners field of view (FOV; 48 cm). Dependent on the femur length of the subject, seven to eight T1-weighted (T1w) axial scans of the femur (TE: 17; TR: 500; matrix: 864×864 ; FOV: 4,200) with a slice thickness of 10 mm and an interslice gap of 50 mm was performed. The first slice was always positioned just below the femur condyles, where the tibia plateau was visible to ensure the same scan position between examination time points. Quadriceps muscle CSA was analyzed as described in detail elsewhere (73). The axial slice equivalent to 50% of the femur length was used to calculate the CSA, and the measurements were performed three times by a blinded, trained person using the imaging software Osirix 2.7.5 (Osirix Imaging Medical, Geneva, Switzerland). The mean value of the three measurements was used as the result, and the coefficient of variation for acceptance between measurements was set to $<2.5\%$.

Tendon CSA. The patellar tendon was scanned in the same 1.5-T MRI scanner as above with the patient supine using a dedicated knee coil using an axial and sagittal T1w turbo spin echo sequence (TE: 17; TR: 500; matrix: 512×512 ; FOV: 150 mm; slice thickness: 3 mm). The knee was slightly flexed (12%) due to the build-in supportive pillow in the coil, which ensured removal of slack in the patellar tendon. The axial slices of the patellar tendon were positioned orthogonal to the length in the sagittal plane covering the distal patellar pole to the tibial insertion. The procedure for analyzing the patellar tendon has been described in detail elsewhere (16, 40). In brief, patellar tendon CSA was measured using the axial slice just distal to the patellar insertion, and the slice just proximal to the tibia insertion and midway between these two sites, respectively (16, 40). The patellar tendon length was obtained by measuring the distance from the most dorsal insertion part at the patella apex to the dorsal insertion on the tibia. Patellar tendon CSA and length were manually outlined by a blinded examiner using the imaging software Osirix 2.7.5 (Osirix Imaging Medical), as described in detail elsewhere (16, 40). All measurements were performed three times by the same observer, who was blinded to the chronological MRI time points and treatment groups (GH or Plc). The mean value of these measurements was used as the result, and the coefficient of variation for acceptance between duplicate measurements was set to $<2.5\%$.

Maximal muscle strength and mechanical properties of patellar tendon. Details of the maximal voluntary contraction (MVC) measurement and analysis of tendon mechanical properties, including reliability assessments, have been reported previously (16, 40). Within-day correlation coefficient and typical error percent results for repeated measures were 0.95 and 9.9% for tendon stiffness, 0.97 and 5.5% for tendon strain, and 0.94 and 9.4% for Young's modulus. The subjects were asked to refrain from strenuous exercise 48 h before the experiments. The subjects performed a 5-min warm-up on a stationary

bike to ensure a standardized preconditioning of the tendon before testing. Subsequently, the subjects were seated in a custom-made rigid chair with both hips and knees flexed to an angle of 90° . A leg cuff, which was connected to a strain gauge (Noraxon) through a rigid steel rod perpendicular to the lower leg, was mounted on the leg just above the medial malleolus. A Hitachi EUB-6500 ultrasound scanner (Hitachi Medical, Tokyo, Japan) equipped with a 10-MHz, 100-mm-long, linear array B-mode transducer (Hitachi, model EUP-L53L) was fitted into a custom-made rigid cast that was secured to the skin above the patellar tendon in the sagittal plane. The ultrasound probe and cast were positioned so that the distal patella, the entire patellar tendon, and the proximal tibia were all visible within the field of view throughout the performed isometric ramp contractions (see below).

The subjects performed four to five slow isometric ramp contractions of the knee extensor by applying gradually increasing force until reaching maximum force (MVC) over a 10-s period, during which patellar tendon displacement and knee extension force were synchronously measured. All measurements were performed unilaterally (immobilized limb). Maximal muscle strength of the quadriceps muscles (MVC) was determined as the peak knee extensor moment produced during the 10-s ramp contraction. During the ramp contractions, ultrasound S-VHS video images were sampled at 25 Hz using frame-by-frame capturing software (Matrox Morphis Dual frame grabber and Matrox Imaging Library software, Matrox Electronic Systems, Montreal, Canada). Custom-made, frame-by-frame tracking software, using a pyramidal implementation of the Lukas-Kanade optical flow estimation, was used to assess the tendon deformation from the ultrasound videos. The accuracy and reproducibility of this tracking software have previously been assessed (47). A trigger signal (Pulse generator, PG 58AA, GouldAdvance, Essex, UK) initiated recording of force and ultrasound video, thus allowing for subsequent synchronization of all recorded data during the ramp contractions. The external tibia moment arm was measured (from the leg cuff to the lateral epicondyle of the knee) to calculate the knee extensor moment ($M = \text{force} \times \text{moment arm}$). The force applied to the patellar tendon was calculated by dividing the measured knee extensor moment by the internal patellar tendon moment arm, which was estimated from individually measured femur lengths (16, 40). Tendon deformation was defined as the change in distance between the patellar apex and tibia (16, 40). Tendon stress was calculated by dividing tendon force with the average tendon CSA (mean of proximal, middle, and distal CSA from MRI). Tendon strain was calculated as the change in length normalized relative to initial resting tendon length. Polynomial functions (second order) were fitted to each single force-deformation curve. Tendon stiffness ($\Delta \text{force} / \Delta \text{deformation}$) and Young's modulus ($\Delta \text{stress} / \Delta \text{strain}$) were calculated at the final 10% of the force-deformation and stress-strain curves, respectively.

All structural measurements [quadriceps muscle CSA and patellar tendon CSA, maximal muscle strength (MVC), and patellar tendon mechanical properties] were performed on both sides at baseline (Pre) and after 6 wk of strength training while only in the immobilized leg after immobilization (Post immob) and at 2 wk of retraining.

Tissue biopsy sampling. Muscle biopsies were obtained at baseline, just after immobilization, and again after 2 and 6 wk of rehabilitation. Tendon biopsies were taken at baseline at the control leg and at the immobilized leg after the immobilization period to avoid potential re-biopsy effects, and again from both sides after 6 wk of retraining. After initiating the present study, we analyzed biopsies from another study and found that a 6-wk "washout period" is not enough to avoid a repeated biopsy effect on mRNA for several growth and matrix factors (like IGF-I and COL) (20). Based on these data, the biopsies from the 6 wk of retraining were not used in the statistical analysis of time or group interactions. The muscle and tendon biopsy procedures have been described in detail elsewhere (30, 38). In brief, the sample sites were prepared after sterilization with local anesthetics (lidocaine, 1%). Muscle tissue was taken from the vastus lateralis muscle using a 5-mm Bergström needle with suction (7), and tendon tissue was

taken from the patellar tendon (Bard Magnum Biopsy Instrument, C.R. Bard, Covington, GA) with a 14-G needle (55). The total wet weight of the muscle sample was 80–100 mg, and the total wet weight of the tendon sample was 8–10 mg. The biopsy samples were cleared of external adipose tissue and blood, frozen in liquid nitrogen, and stored at -80°C for subsequent analysis.

Hormone measurements. Blood samples drawn from the antecubital vein were separated (3,200 g, 4°C), and serum was stored at -80°C . Serum IGF-I (sIGF-I) concentrations were determined in duplicates with GH ELISA, RMEE022 (Biovendor, Heidelberg, Germany), and IGF-1 ELISA, RMEE20 (Biovendor).

sIGF-1 was used as a marker for the effect of GH supplementation since rhGH injected subcutaneously (sGH) has a relative short half-time (2–4 h) compared with sIGF-1 (days) and since normal endogenous human GH is secreted in 6–12 discrete pulses per day, resulting in fluctuating values.

Muscle and tendon mRNA measurements. Tendon and muscle were homogenized in TriReagent (Molecular Research Centre, Cincinnati, OH) using a bead-mixer with steel beads (Biospec Products, Bartlesville, OK). Following homogenization, bromo-chloropropane (Molecular Research Centre) was added to separate the samples into an aqueous and an organic phase. Glycogen was added to the tendon samples to improve RNA precipitation. Following isolation of the aqueous phase, RNA was precipitated using isopropanol, washed in ethanol, and dissolved in RNase-free water. All tissue samples were weighed before RNA extraction. Muscle RNA concentrations were determined by spectroscopy, and tendon RNA concentrations were determined using RiboGreen assay (Molecular Probes, Eugene, OR). Good muscle RNA quality was ensured by gel electrophoresis (tendon samples had too little RNA for gel electrophoresis). The amount of mRNA for collagen I (COL1A1), collagen III (COL3A1), IGF-IEa, IGF-IEc, lysyl oxidase (LOX), MMP-2, MMP-9, decorin, GAPDH, and RPLP0 was determined and measured with reverse-transcription, real-time PCR, as described in detail elsewhere (20). Primer sequences are given in Doessing et al. (20), except for LOX (CGC TGT GAC ATT CGC TAC ACA GGA C, CAT TGG GAG TTT TGC TTT GCC TTC T), MMP-2 (CCG CCT TTA ACT GGA GCA AAA ACA, TTG GGG AAG CCA GGA TCC ATT T), MMP-9 (AGC GAG GTG GAC CGG ATG TT, AGA AGC GGT CCT GGC AGA AAT AG), and decorin (GGT GGG CTG GCA GAG CAT AAG T, TGT CCA GGT GGG CAG AAG TCA). RPLP0 was used for normalization. To validate RPLP0 as an unregulated mRNA useful for normalization, we also measured GAPDH. Ideally, the ratio between GAPDH and RPLP0 should be one in all groups. Unfortunately, after immobilization, there was a slight decrease in GAPDH relative to RPLP0 in the placebo group, indicating that either GAPDH decreased or RPLP0 increased at that specific time point (Fig. 2). It is not possible to distinguish between the two possibilities, but during immobilization we find it more likely that the metabolic marker GAPDH decreased than protein synthesis (RPLP0) increased, for which reason RPLP0 was chosen for normalization.

Transmission electron microscopy imaging and stereology analysis (tendon). The procedure from the tendon biopsies for making transmission electron microscopy (TEM) images for stereologic analyses of tendon fibrillar morphology has previously been described in detail (39). In brief, after tendon biopsies were rinsed in 0.15 M sodium cacodylate buffer (pH 7.2), all specimens were fixed in 1% OsO_4 in 0.15 M sodium cacodylate buffer (pH 7.2) for 2 h and subsequently dehydrated in ethanol, transferred to propylene oxide, and embedded in EPON resin 828/862 mix (Hexion Specialty Chemicals, Rotterdam, The Netherlands), according to standard procedures. After this, sections were cut using a Reichert-Jung Ultracut E microtome. Semi-thin sections, for preanalytic evaluation of the samples, were stained with toluidine blue and visualized (digital images) using a Nikon Coolpix 990 (Nikon Nordic ABL, København, S. Denmark). When proper condition and orientation of the sample within the EPON block were confirmed, ultra-thin sections were cut and collected on a one-hole

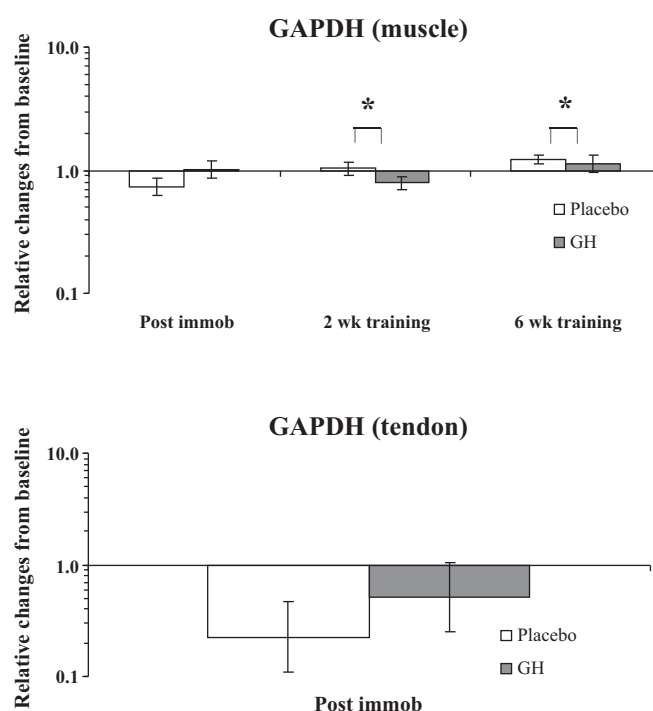


Fig. 2. Muscle and tendon GAPDH mRNA expression after 2 wk of immobilization and 6 wk of retraining in elderly men ($n = 12$) with either rhGH ($n = 6$) or placebo ($n = 6$). A: GAPDH mRNA (muscle). B: GAPDH mRNA (tendon). Data are geometric means \pm SE. *Significant time effect difference compared with baseline (both groups; $P < 0.05$).

copper grid with Formvar supporting membranes and stained with uranyl acetate and lead citrate. From each biopsy sample cross section, a simple random sample of 10 digitized TEM images was obtained from the intercellular space.

The stereologic analyses of the TEM images were completed on a computer monitor, onto which the digitized TEM images were merged with a graphic representation of the stereologic test system (C.A.S.T.-grid software, The International Stereology Centre at Olympus). The collagen fibrils were counted and measured using a magnification of 210,000. The counting frames covered 3% of the TEM image area, and the TEM images covered $\sim 0.5\%$ percent of the biopsy sample cross section ($\sim 5 \mu\text{m}^2$). On average, 455 fibrils (range, 150–1,209) were analyzed per cross section. Fibril area was calculated from measurements of fibril diameter. Fibril diameter was measured as the largest diameter perpendicular to the longest axis of each fibril cross section, thereby eliminating the influence of sectioning angle. The fibril density was expressed as the absolute number of fibrils per square micrometer, and the fibril volume fraction states the area occupied by fibrils within the sample area. A single experienced and blinded investigator (J. O. Larsen) performed all stereological analyses.

Statistics Analysis

Data were analyzed in SigmaPlot version 11 using two-way, repeated-measures ANOVA with Student-Newman-Keuls post hoc test. Data are presented as means \pm SE. mRNA data from RT-qPCR and serum IGF-1 (sIGF-1) levels were log transformed before the statistical analysis and presented as geometric means \pm back-transformed SE. An alpha level of $P < 0.05$ was considered significant.

Due to technical problems, biomechanical data could only be obtained for four participants in Plc group post-immob and after 2 wk of retraining.

RESULTS

Subjects

All 12 participants completed the study and complied with all procedure in regard to rhGH/Plc administration according to diaries and sGH/IGF-1 measures, immobilization, and training sessions.

At baseline, there was no significant difference between the two groups in age (means \pm SE: GH: 72.5 \pm 2.2 yr; Plc: 70.2 \pm 3.6 yr); height (GH: 177 \pm 2 cm; Plc: 177 \pm 2 cm), weight (GH: 76.0 \pm 2.4 kg; Plc: 79.9 \pm 4.4 kg), or body mass index (23.5 \pm 0.7 kg/m²; Plc: 24.1 \pm 3.4 kg/m²).

Body Composition

Body weight (BW), total fat mass (FM), or total lean body mass (LBM) remained stable throughout the study in the Plc group (Table 1). In the GH group, although BW was unchanged, a decrease in FM was observed along with an increase in LBM (baseline vs. post-intervention, $P < 0.05$; Table 1).

sIGF-1

As a result of the rhGH supplementation, a time and group interaction was seen for sIGF-1 ($P < 0.001$; Fig. 3). There were no significant differences between groups in sIGF-1 at baseline. However, sIGF-1 increased approximately fivefold during immobilization, and an approximately fourfold increase was maintained during retraining in the GH group, with no changes observed in the Plc group (Fig. 3).

Tendon CSA and Biomechanical Properties

A time effect and a group interaction were demonstrated for patellar tendon CSA ($P < 0.05$), with no group differences for patellar tendon CSA before immobilization (baseline) or during immobilization (Fig. 4A). However, patellar tendon CSA increased in both groups during retraining compared with baseline ($P < 0.05$), with a significantly larger increase in the GH group compared with the Plc group after 6 wk of retraining (GH: 7.7 \pm 1.1% vs. Plc: 2.9 \pm 1.1%; $P < 0.05$; Fig. 4A). Patellar tendon stiffness revealed no overall time effect, but a

Table 1. Changes in body composition (DEXA) baseline vs. postintervention (2 wk of immobilization and 6 wk of rehabilitation)

Changes in body composition	GH (n = 6)		Plc (n = 6)	
	Mean	SE	Mean	SE
Weight (kg), baseline	76.0	2.4	79.9	4.4
Weight (kg), postintervention	77.0	1.9	80.7	4.4
% Fat total, baseline	23.5	0.7	24.2	3.4
% Fat total, postintervention	19.4*	1.2	25.1	3.2
Total FM (kg), baseline	17.1	0.7	19.0	3.4
Total FM (kg), postintervention	15.4*	1.4	20.1	3.3
Total LBM, baseline	55.7	1.8	57.8	1.5
Total LBM, postintervention	59.3*	1.2	57.5	1.2

Changes in body composition (DEXA) baseline vs. postintervention (2 wk of immobilization and 6 wk of rehabilitation) in elderly men ($n = 12$) with either recombinant human growth hormone (rhGH; $n = 6$) or placebo (Plc; $n = 6$). All values are means \pm SE. GH, growth hormone group; FM, fat mass; LBM, lean body mass. Post intervention = 2 wk of immobilization + 6 wk of retraining. *Significant time effect difference compared with baseline ($P < 0.05$).

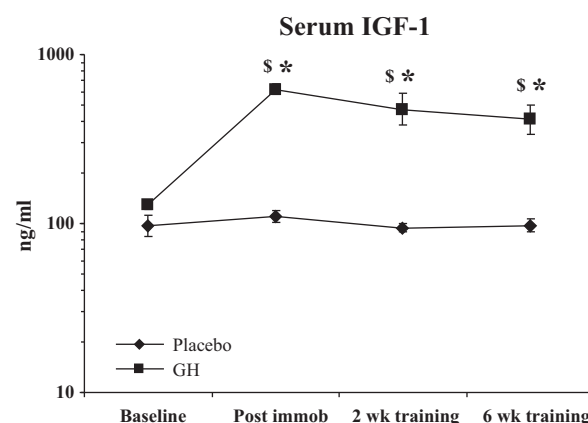


Fig. 3. Circulating serum concentrations of IGF-1 after 2 wk of immobilization and 6 wk of retraining in elderly men ($n = 12$) with either rhGH ($n = 6$) or placebo ($n = 6$). Data are geometric means \pm SE. *Significant time effect difference compared with baseline ($P < 0.001$). †Significant group effect of GH vs. placebo within time point ($P < 0.01$).

group interaction was seen ($P < 0.05$). During immobilization, no changes in tendon stiffness emerge within any of the groups, but, after retraining, tendon stiffness was lower in the Plc group compared with the GH group, with a significant difference between groups after 6 wk of retraining ($P < 0.05$; Fig. 4B). Young's modulus for the patellar tendon showed no overall time effect, but a tendency toward a differential retraining response of reduced modulus in Plc compared with GH was observed ($P = 0.07$; Fig. 4C). All other tendon biomechanical parameters (tendon deformation, strain, or stress throughout the immobilization and retraining period) were unchanged in both groups (Table 2).

Tendon Fibrillar Morphology

Fibril volume fraction, fibril density, mean fibril diameter, and mean fibril area did not differ between GH or Plc at any time points and did not change over the time course of immobilization and retraining (Fig. 5, A–D).

Quadriceps Muscle CSA and Maximal Muscle Strength

After immobilization, quadriceps muscle CSA decreased more in Plc compared with GH (GH: $-4.1 \pm 1.9\%$; Plc: $-9.2 \pm 1.8\%$; $P < 0.05$; Fig. 6A). During retraining, the quadriceps muscle CSA returned to baseline values in both groups already after 2 wk of training, with a further increase in GH compared with Plc after 6 wk of training (GH: $9.4 \pm 2.1\%$; Plc: $3.1 \pm 1.3\%$, relative to baseline; $P < 0.05$; Fig. 6A). At baseline, there was no difference in quadriceps strength between the GH and Plc groups. After 2 wk of immobilization, maximal quadriceps strength was reduced to the same degree in both groups (GH: $-39 \pm 4.7\%$; Plc: $-29 \pm 5.9\%$; $P < 0.01$; Fig. 6B), and both groups regained their muscle strength during retraining, with no differences between groups (Fig. 6B).

Muscle and Tendon Extracellular Matrix mRNA IGF-1Ea/Ec and Collagen 1A1/3A1 Expression

Muscle. Both a time effect and a group interaction were seen in the skeletal muscle gene expression ($P < 0.001$). IGF-1Ea and IGF-1Ec mRNA increased after 2 wk of immobilization and subsequent retraining in the GH group, which demon-

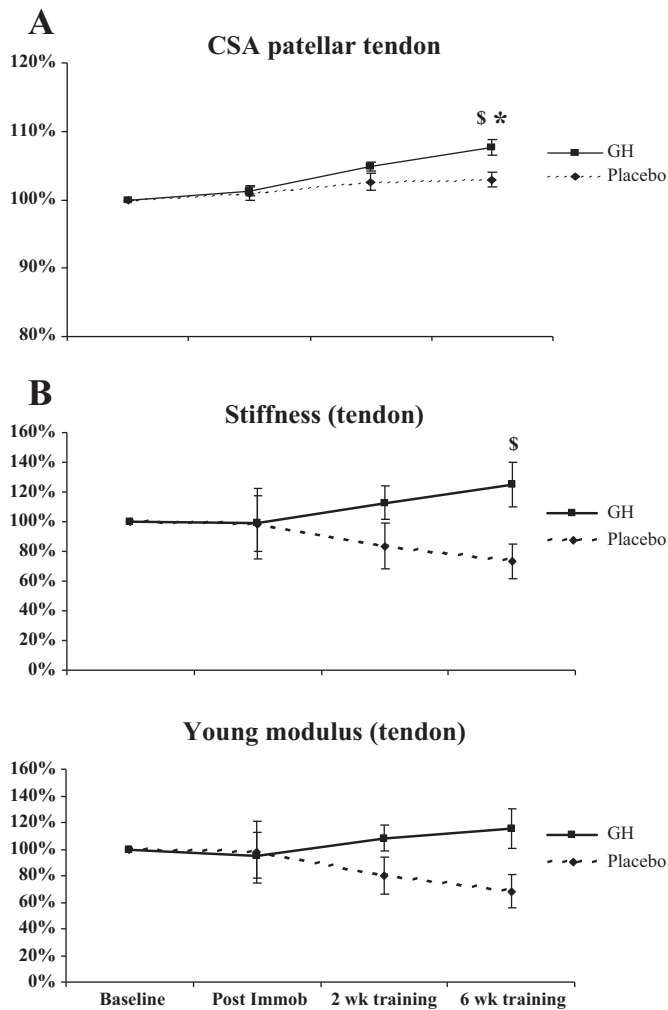


Fig. 4. Changes in patellar tendon CSA and biomechanical properties following 2 wk of immobilization and 6 wk of retraining in elderly men ($n = 12$) with either rhGH ($n = 6$) or placebo ($n = 6^a$). A: patellar tendon CSA, time effect ($P < 0.05$); group interaction ($P < 0.05$). B: patellar tendon stiffness, no overall time effect; group interaction ($P < 0.05$). C: patellar tendon Young modulus, no overall time effect; group interaction ($P = 0.07$). Data are means \pm SE. *Significant time effect difference compared with baseline (both groups; $P < 0.05$). §Significant group effect difference between GH and Plc within time point ($P < 0.05$). ^aBiomechanical data (stiffness and modulus) could only be obtained for four participants in Plc group post-immob and after 2 wk of training.

strated greater changes than Plc ($P < 0.01$; Fig. 7, A and B). There were no changes in IGF-1Ea and IGF-1Ec in Plc following immobilization, but increased IGF-1Ec mRNA was seen after 6 wk of retraining ($P < 0.05$; Fig. 7A). Relative to

baseline and Plc, GH supplementation increased mRNA COL1A1 and COL3A1 in muscle following immobilization and during retraining ($P < 0.01$; Fig. 7, C and D).

Tendon. No time or group interactions were observed for tendon IGF-1Ea, COL1A1, and COL3A1 mRNA expression after 2 wk of immobilization with GH supplementation or Plc supplementation, respectively (Fig. 7, E–G). IGF-1Ec mRNA concentration was below the detection limit in the majority of the tendon biopsies and no further analysis was made on the samples. Furthermore, no significant differences in LOX, MMP-2, MMP-9, and decorin mRNA expression were observed compared with baseline or between the groups following the 2-wk immobilization period (Fig. 8, A–D). Due to the effect of repeated biopsies on tendon, no further analysis was made on mRNA expression during the retraining phase.

DISCUSSION

The main findings in the present study were that GH administration stimulated both local IGF-1 and collagen expression in skeletal muscle of elderly individuals, which likely contributed the greater tendon stiffness observed in GH-supplemented individuals compared with nonsupplemented individuals following 2 wk of lower limb immobilization succeeded by 6 wk of rehabilitation. This effect was present despite the lack of a differential effect of GH supplementation on muscle strength during the same time period.

GH and Changes in Tendon Tissue

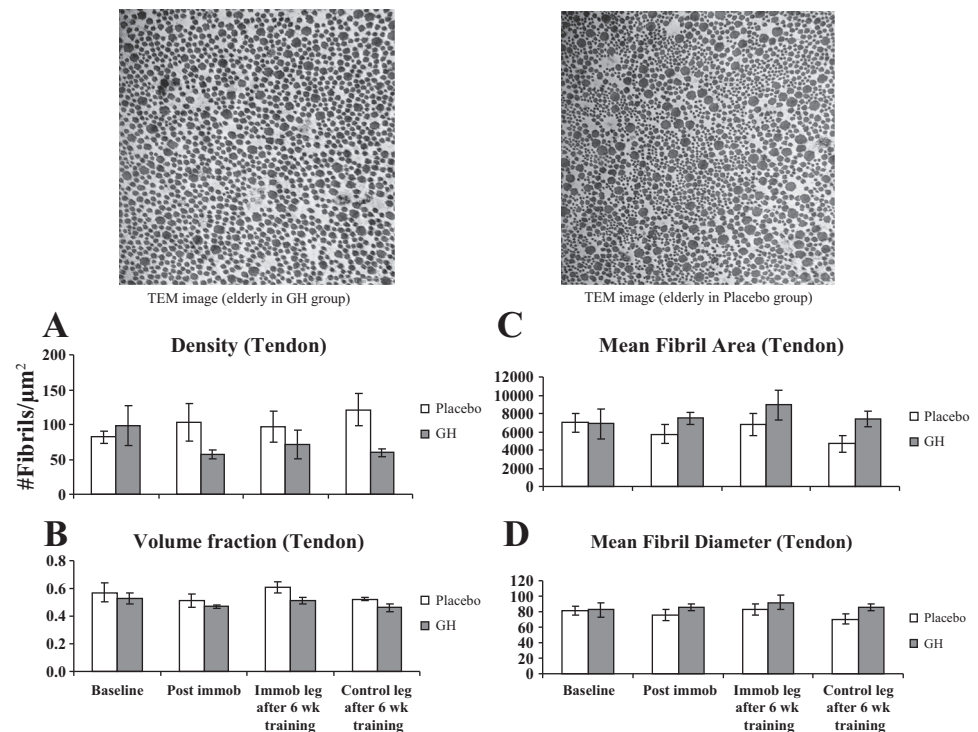
In the present study, no significant change in tendon size was detected with immobilization, and a rise was found during rehabilitation, and this rise was more pronounced during rehabilitation in the presence of GH administration (GH: 8%; Plc: 3%; Fig. 4A). The fact that an effect of immobilization could not show any change in tendon size is in agreement with recent human studies in young and elderly (14, 17, 70). However, due to the small sample size, it cannot fully be excluded that an effect of immobilization upon tendon size, fibril size or density could have been missed. The increase in tendon size in response to subsequent reloading also conforms to previous findings, where long-term strength training or habitual loading appears to be associated with an increase in tendon size (16, 40, 68). In addition to a positive effect on tendon size following the period of rehabilitation, GH also led to altered biomechanical tendon properties reflected by elevated tendon stiffness, with GH supplementation vs. no supplementation (Fig. 4B). The difference in stiffness and modulus between the two groups following rehabilitation (Fig. 4, B and C) is likely to be explained by GH administration leading to increased tendon

Table 2. Changes in tendon biomechanical properties during immobilization and rehabilitation

	GH ($n = 6$)								Plc ($n = 6$)							
	Baseline	2 wk immob	Δ , %	2 wk train	Δ , %	6 wk train	Δ , %		Baseline	2 wk immob	Δ , %	2 wk train	Δ , %	6 wk train	Δ , %	
Deformation, mm	1.85 \pm 0.2	1.74 \pm 0.2	0.3 \pm 12.4	1.83 \pm 0.2	2.8 \pm 12.4	1.52 \pm 0.1	-14.1 \pm 7.8		1.45 \pm 0.1	1.52 \pm 0.2	9.5 \pm 19.4	1.62 \pm 0.2	17.9 \pm 23.6	1.74 \pm 0.2	23.9 \pm 16.1	
Strain, %	4.3 \pm 0.5	4.1 \pm 0.4	3.4 \pm 13.0	4.2 \pm 0.5	2.0 \pm 12.4	3.5 \pm 0.3	-13.5 \pm 7.8		3.25 \pm 0.4	3.5 \pm 0.5	13.5 \pm 20.4	3.9 \pm 0.5	22.2 \pm 23.9	4.0 \pm 0.5	27.4 \pm 19.5	
Stress, MPa	20.1 \pm 2.3	20.1 \pm 2.3	0.3 \pm 2.5	19.7 \pm 2.0	-1.3 \pm 1.9	19.3 \pm 2.2	-3.6 \pm 2.3		22.4 \pm 2.7	22.7 \pm 2.7	1.5 \pm 1.9	22.2 \pm 2.9	-1.9 \pm 1.5	21.3 \pm 2.7	-5.0 \pm 2.3	

Tendon biomechanical properties following 2 wk of immobilization and 6 wk of retraining in elderly men ($n = 12$) with either rhGH ($n = 6$) or Plc ($n = 6$). All values are means \pm SE. 2 wk immob, 2 wk of immobilization; 2 wk train, 2 wk of retraining; 6 wk train, 6 wk of retraining; Δ , average of relative changes (%). Biomechanical data could only be obtained for four participants in the Plc group postimmob and after 2 wk of training. *Significant time effect difference compared with baseline ($P < 0.05$). §Significant group effect difference between GH and Plc within time point ($P < 0.05$).

Fig. 5. Changes in patellar tendon fibril morphology after 2 wk of immobilization and after 6 wk of retraining in elderly men ($n = 12$) with either rhGH ($n = 6$) or placebo ($n = 6$). A: tendon fibril density (no./ μm^2). B: volume fraction (tendon). C: tendon mean fibril area (nm^2). D: tendon mean fibril diameter (nm). No time effect or group interactions were seen within time points. Data are means \pm SE.



CSA over the entire study period (Fig. 4A). The mechanism behind this effect of GH on tendon CSA and stiffness during rehabilitation is presently unknown but might be due to the fact that GH increases IGF-1 levels and that local IGF-1 and

collagen expression may have been stimulated in the tendon during the phase of retraining, as previously demonstrated in young healthy men (20). Due to a repeated biopsy effect, which yields substantial amounts of gene upregulation due to the procedure of biopsy sampling itself, it was not possible to analyze tendon IGF-1 and collagen expression during the rehabilitation phase. Notably, fibril size or density did not change during the intervention (Fig. 5). This could indicate that the enlargement of the tendon CSA with GH is due to accumulation of other extracellular matrix substances or that the GH led to neoformation of collagen fibrils with morphological characteristics that on average resembled the fibrils already present within the tendon. Regardless, the findings from the present study suggest that an increase in IGF-1 level in elderly via GH administration (Fig. 3) stimulates tendon tissue proliferation (Fig. 4A), which indicates that the GH/IGF-I axis likely was involved in the concurrent adaptation in tendon mechanical properties (Fig. 4, B and C).

The reduced response in tendon stiffness in the Plc group compared with the GH group is in accordance with a recent study on young men (9). The study on young men showed that GH administration maintained both tendon stiffness and modulus compared with control during a period of inactivity (9). Furthermore, tendon stiffness increased with GH, with no changes in Plc during 6 wk of retraining together with a larger increase in tendon CSA size (GH: 16% vs. Plc: 11%) (9). The reason for this effect of GH on tendon stiffness is presently unknown. Alterations in collagen cross-linking as a result of an increase in the expression of the enzyme lysyl oxidase (LOX) within the tendon have been suggested in young individuals (9). Cross-links are a major contributor in stabilizing collagen molecules that positively affects intermolecular force transduction and therefore play an important role in mechanical properties of the tendon (4, 5). Two types of cross-links have been

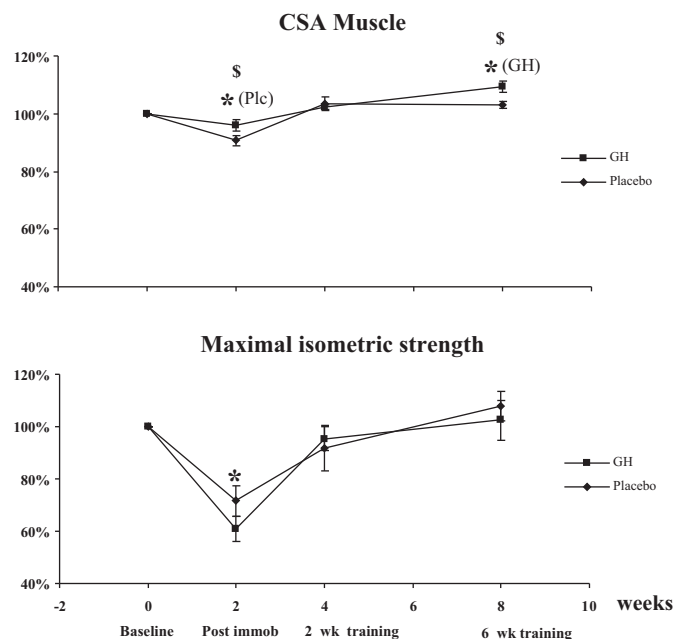


Fig. 6. Changes in quadriceps muscle CSA and maximal isometric strength following 2 wk of immobilization and 6 wk of retraining in elderly men ($n = 12$) with either rhGH ($n = 6$) or placebo ($n = 6$). A: quadriceps muscle CSA, time effect ($P < 0.01$); group interaction ($P < 0.05$). B: maximal isometric muscle strength, time effect ($P < 0.001$); no overall group interaction. Data are means \pm SE. *Significant time effect difference compared with baseline ($P < 0.05$). \$Significant group effect difference between GH and Plc within time point ($P < 0.05$).

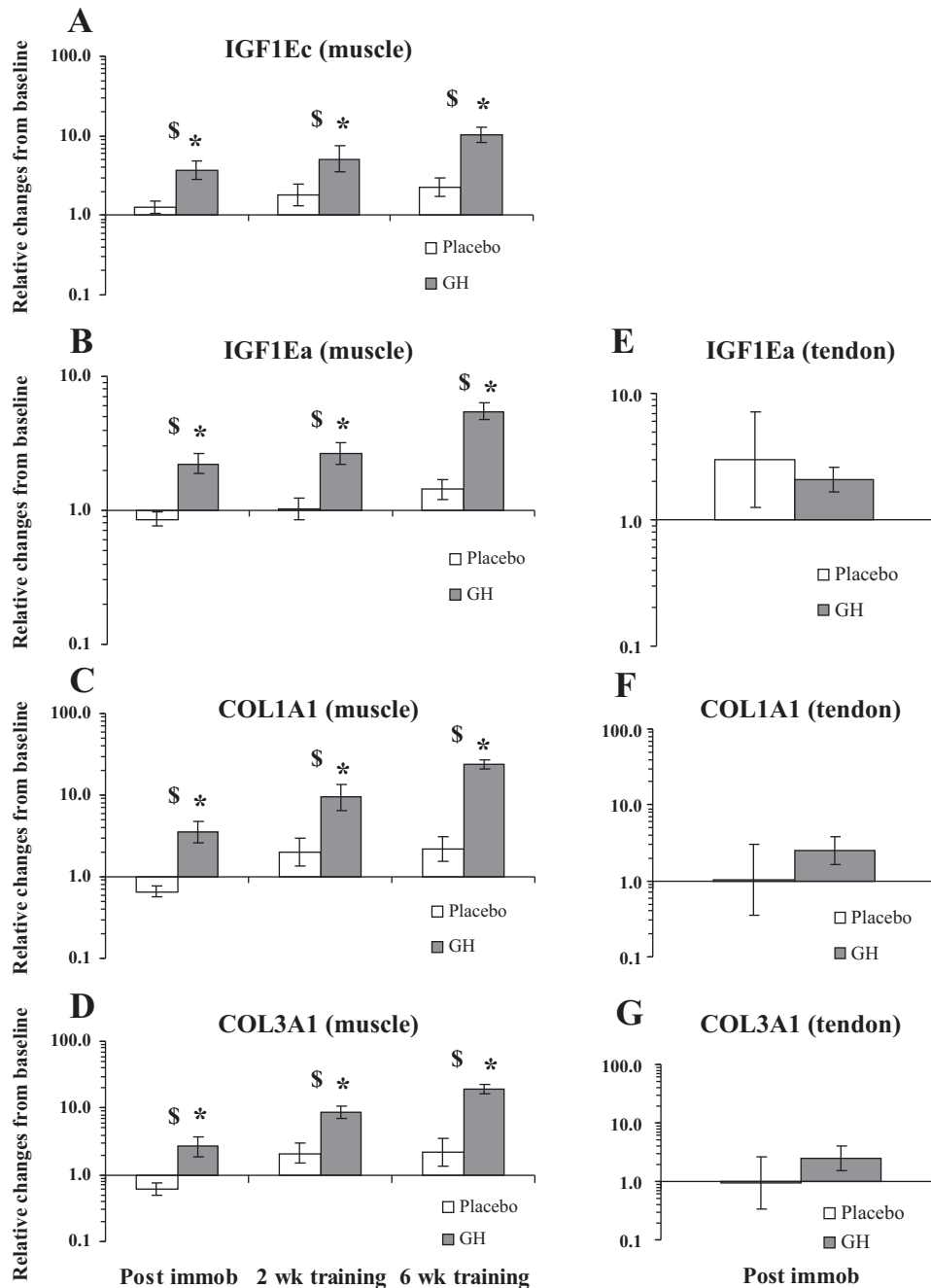


Fig. 7. Skeletal muscle and tendon IGF-1 and collagen (COL) mRNA expression following 2 wk of immobilization and 6 wk of retraining (muscle) in elderly men ($n = 12$), with either rhGH ($n = 6$) or placebo ($n = 6$). A: IGF-1Ea mRNA (muscle), time effect ($P < 0.001$); group interaction ($P < 0.001$). B: IGF-1Ec mRNA (muscle), time effect ($P < 0.001$); group interaction ($P < 0.001$). C: COL 1A1 mRNA (muscle), time effect ($P < 0.001$); group interaction ($P < 0.001$). D: COL 3A1 (muscle), time effect (0.001); group interaction (0.001). E: IGF-1Ea mRNA (tendon), no overall time effect or group interaction ($P < 0.05$). F: COL 1A1 mRNA (tendon), no overall time effect or group interaction. G: COL 3A1 (tendon), no overall time effect or group interaction. Data are geometric means \pm SE. *Significant time effect difference compared with baseline ($P < 0.01$). \$Significant group effect between GH and Plc within time point ($P < 0.01$).

described, enzymatic and nonenzymatic cross-links (4, 5), and LOX is known to be associated with the formation of enzymatic cross-links (60, 71), which has been shown to be increased by GH supplementation in young men (9). However, no longitudinal changes in LOX were observed in the present study (Fig. 8A). Again, it has to be underlined that only a limited number of subjects participated in the present study, and it cannot be precluded that the large scatter in the data is limiting a solid conclusion. Increased IGF-I levels have been shown in other experiments to be associated with an overexpression of LOX along with an increase in enzymatic cross-link formation (61). In a study on rat vascular cells grown on matrices, IGF-I (plus hyaluronic acid fragments) resulted in an increased density of cross-links within cell layers (43). Fur-

thermore, that notion of IGF-I is indirectly supported by a study in experimental rats, where application of a mixture of IGF-I and FGF-2 resulted in increased LOX expression in the oral connective tissues (77).

GH and Changes in Muscle CSA

In the present study, muscle CSA declined in the Plc group during immobilization, which was not the case when GH was administered (GH: -4% vs. Plc: -9% ; Fig. 6A). Even though no significant decrease in muscle size was seen in GH during immobilization, maximal muscle strength declined to a similar extent in both groups (GH: -39% , Plc: -29% ; Fig. 6B). Decreases in muscle mass and muscle strength during short

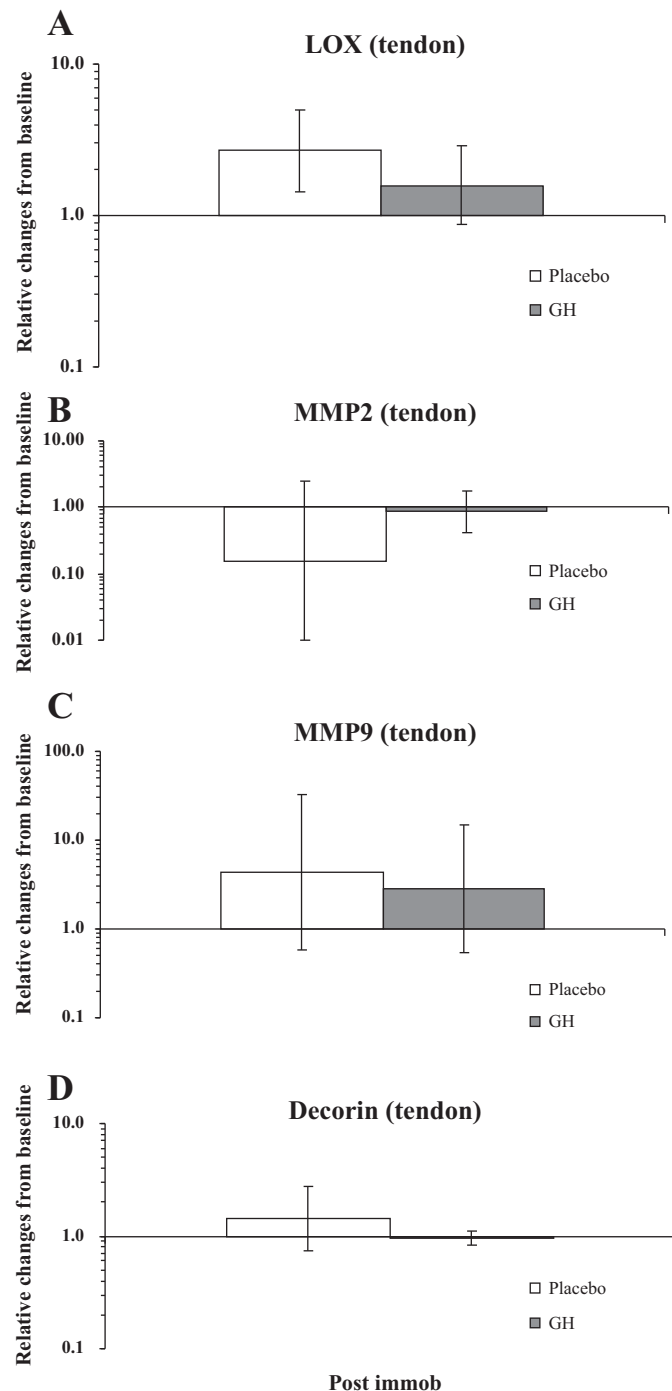


Fig. 8. Tendon LOX, MMP-2 and -9, and decorin mRNA expression following 2 wk of immobilization in elderly men ($n = 12$), with either rhGH ($n = 6$) or placebo ($n = 6$). A: LOX mRNA (tendon). B: MMP-2 mRNA (tendon). C: MMP-9 mRNA (tendon). D: decorin mRNA (tendon). Data are geometric means \pm SE. No overall time effect or group interaction were seen within time points.

periods (weeks) of inactivity have previously been reported in elderly individuals (32, 41, 42, 73). A possible explanation for the greater loss of muscle strength compared with muscle mass observed in the present study could be attributed by a decline in neuromuscular activity (67, 73). However, we did in the present study not determine the neuromuscular activity. Fur-

thermore, we did only determine the anatomical CSA and not the physiological CSA, a parameter which is better correlated to the strength than the anatomical CSA (12, 57). During the subsequent phase of rehabilitation, both groups regained muscle mass and muscle strength, with a greater increase in muscle CSA observed with GH supplementation (GH: 9% vs. Plc: 3%; Fig. 6A). Recent studies have indicated that elderly individuals may have a reduced ability to regain the loss in muscle mass induced by short-term immobilization during the subsequent phase of retraining (32, 73). This was, however, not the case in the present study, where the participants in both the GH and the Plc groups demonstrated a full recovery in muscle CSA and contractile capacity (MVC) during the 6 wk of retraining. These disparate findings could arise from study differences in the duration of immobilization and retraining, respectively, and/or different muscle assessment procedures employed [anatomical muscle CSA obtained by MRI (present study) vs. myofiber CSA obtained by muscle biopsy sampling (32, 73)]. Furthermore, possible differences in the physical activity status of the experimental subjects (i.e., different levels of inactivity before inclusion) may have resulted in different study outcomes.

As one of the important findings in the present study, the loss of skeletal muscle mass in response to inactivity in the elderly seems to be attenuated by administration of GH in the present study (Fig. 6A). This indicates that increased GH/IGF-I axis activity via elevated local IGF-I inhibits the effect of inactivity on skeletal muscle in elderly subjects. However, since no difference in maximal muscle strength could be detected between GH-supplemented and nonsupplemented subjects, it cannot be excluded that the enhanced gain in muscle size observed in the GH group could be due to intramuscular water retention rather than to muscle growth per se, as previously described (15, 44, 63). This notion is supported by other human studies, where no effect of GH on myofibrillar protein synthesis was found (20, 44, 79). However, yet other studies have demonstrated GH administration in elderly individuals to result in increased muscle volume (63, 78) or improved mechanical muscle function (44, 58, 63, 74). At present, only a single study in healthy elderly subjects has reported muscle mass and muscle strength to increase after GH administration (63, 78). GH has earlier been used in intensive care patients, causing protein retention both in patients receiving low-dose GH (23, 82) and supraphysiological GH doses (76, 83). The positive effect on muscle protein synthesis is supported by a study in elderly women receiving short-term GH or IGF-1 treatment resulting in an elevated muscle protein synthesis and increased net whole body protein synthesis (11). These findings indicate that GH may in part counteract the net muscle loss induced by short-term periods of inactivity. Nevertheless, since muscle strength was not influenced by GH in the present and others studies (44, 63, 74), the possibility exists that the present superior gains in muscle CSA with GH may mainly have been due to fluid retention, which is a known side effect by GH supplementation (15, 44, 63).

GH and Body Composition

In the present study, 8 wk of GH supplementation led to decreases in total FM by 10% and LBM by 7%, with no changes seen in the Plc group (Table 1). These findings are in

accordance with previous reports showing that GH administration reduces total fat mass and increases lean body mass (45, 58, 63). GH seems to be a strong lipolytic agent (37, 54), and GH administration has been found to reduce total FM in GHD patients (13, 66), young obese men (36, 51), and elderly healthy men (8, 29, 58). DXA-based methods used for determining changes in lean body mass are not capable of distinguishing fluid retention from myofibrillar muscle tissue accumulation, and therefore we cannot rule out that the present increases in LBM observed with retraining and concurrent GH administration were at least in part due to fluid retention. Indirectly supporting this notion, increases in lean body mass evoked by 12 wk of GH administration in young healthy men were found to rapidly disappear after 9 days of GH discontinuation (45).

GH and mRNA Extracellular Matrix Expression in Skeletal Muscle and Tendon Connective Tissue

GH administration was associated with elevated local IGF-I expression and collagen expression in skeletal muscle during the periods of inactivity and rehabilitation (Fig. 7, A–D). This association between GH and local IGF-I/collagen expression failed to be observed in the patellar tendon, which is probably due to large variation in the limited samples obtained (Fig. 7, E–G). Furthermore, it has to be noted that, in the present study, we only determined mRNA expression and not the local protein concentration of IGF-I, and that we did not measure IGF-I binding proteins. Thus we are not able to directly evaluate the local concentration of free IGF-I. Previous animals studies have demonstrated GH to stimulate IGF-I and collagen expression in connective tissue, where changes in the level of physical activity were associated with a change in IGF-I and collagen expression (31). Likewise, in humans, it has been found that GH administration led to increased expression of both IGF-I and collagen in muscle and tendon, which occurred in a parallel manner (20). This is further supported by recent data in young healthy men where GH administration clearly leads to increased IGF-I and collagen expression in the musculo-tendinous tissue during short-term immobilization and retraining (9).

In conclusion, GH administered in healthy, elderly individuals appears to attenuate muscle volume loss during short-term immobilization and stimulates muscle growth during subsequent retraining due to either water retention or muscle tissue maintenance. At the same time, GH administration was found to stimulate collagen expression, tendon size, and stiffness during the rehabilitation phase following short-term inactivity. The present data indicate that GH can influence the connective tissue metabolism in musculo-tendinous tissue and tendon biomechanical properties during short-term immobilization and subsequent rehabilitation in elderly humans.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.P.B., M.K., and H.L. conception and design of research; A.P.B., K.J.D., and M.B. performed experiments; A.P.B., K.J.D., C.C., S.P.M., and P.S. analyzed data; A.P.B., C.C., S.P.M., P.S., P.A., M.K., and H.L. interpreted results of experiments; A.P.B. prepared figures; A.P.B., M.K., and H.L. drafted manuscript; A.P.B., K.J.D., C.C., S.P.M., P.S., M.B., P.A., M.K., and H.L. edited and revised manuscript; A.P.B., K.J.D., C.C., S.P.M., P.S., M.B., P.A., M.K., and H.L. approved final version of manuscript.

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